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In order to determine the structure of the transmembrane domain of the neu/erbB-2 receptor and address the molecular mechanism of receptor activation by the transforming V_{664} to E_{664} mutation, magic angle spinning NMR and polarized Fourier transform infrared studies have been undertaken. These studies show that the region C-terminal to position 664 is helical and oriented roughly perpendicular to the membrane plane. When the E_{664} carboxyl group is deprotonated, the region N-terminal to position 664 unfolds and the COO- group is exposed to the polar membrane interface. Protonation allows the peptide to adopt a helical conformation oriented perpendicular to the membrane plane. The pKa of the E_{664} side chain is shifted by the membrane surface charge. Under conditions approximating those in native membranes, the carboxyl group readily partitions into the membrane. The high pKa observed for the E_{664} carboxyl group and increased orientation in DMPC:DMPS membranes argues that the V_{664} to E_{664} mutation causes the transmembrane domain to dimerize.

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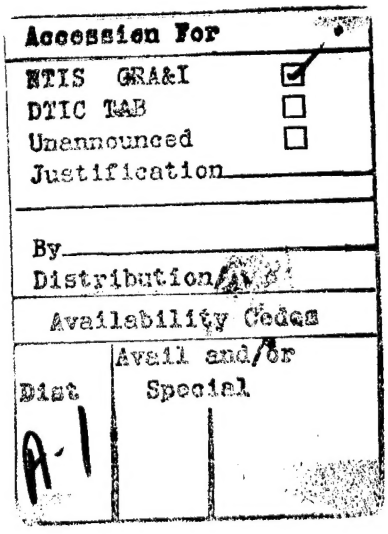

TABLE OF CONTENTS

Rotational Resonance NMR Structural Studies of the neu Receptor Transmembrane Domain

INTRODUCTION

The neu/erbB-2 receptor has two cysteine-rich extracellular domains, a single helical membrane-spanning domain and an intracellular tyrosine kinase domain [1-4]. A single mutation (V664E) within the hydrophobic transmembrane sequence leads to constitutive activation of the receptor.

Two models have been proposed for how the $V_{664} \rightarrow E_{664}$ substitution causes cell transformation. The first involves a change in the secondary structure of the TM domain. Brandt-Rauf et al. (1990) [5] calculated that the minimum-energy conformation in the region of V_{664} contains a sharp bend at positions 664 and 665, while the transforming sequence exists as an α-helix. The second model is that the glutamate side chain promotes dimerization via hydrogen-bonding interactions [6]. In this case, the helices are thought to be held in the "active" state by hydrogen bonding between the E_{664} carboxylic acid side chain on one helix and the E_{664} side chain or peptide backbone C=O/NH groups of the second helix. Consistent with this model, the transforming (E_{664}) protein has a higher propensity to form dimers than the normal (V_{664}) protein [7]. The position of the glutamic acid residue is sequence specific. Bargmann and Weinberg originally demonstrated that substitution of glutamic acid at positions 663 or 665 did not activate the receptor [1].

Stern and coworkers have undertaken an extensive mutagenesis study of the *neu* TM domain to define the residues that are responsible for receptor activation [4]. They found that a sub-domain, $-V_{663}-E_{664}-G_{665}-$, is important. The domain differs slightly from that predicted by Sternberg and Gullick [6], most notably in the importance of A_{661} for receptor dimerization. Gullick and coworkers [8] have shown that intracellular expression of small proteins, corresponding to the TM and membrane-proximal domains of neu and containing variations on the activated neu TM sequence, inhibits growth of neu transformed cells. However, a simple model involving hydrogen-bonding interactions of the E_{664} may not be sufficient to explain receptor activation. Stern and coworkers [4] have shown, by moving the VEG sub-domain around in the TM sequence and by generating mutants that dimerize without activating the receptor, that dimerization alone is insufficient to cause cell transformation and that the interactions are likely to be highly specific. No direct evidence has been obtained for the points of contact in the receptor dimers and a high-resolution structure is needed to determine the exact nature of the protein interactions and whether they are distributed along the length of the TM domain.

In order to determine the structure of the transmembrane domain of the neu/erbB-2 receptor and address the molecular mechanism of receptor activation by the transforming V_{664} to E_{664} mutation, magic angle spinning (MAS) NMR and polarized Fourier transform infrared (FTIR) studies have been undertaken. We have previously shown that MAS NMR and FTIR measurements provide a feasible approach for structural studies on membrane proteins [9-11].

MAS yields high resolution NMR spectra of membrane proteins in bilayer environments [6] and several different strategies have been developed for measuring weak dipolar couplings in MAS experiments [12-15]. Of importance for structural studies is that accurate internuclear distances can be derived from measurements of dipolar couplings which in turn provide constraints for generating and evaluating structural models. The two best established approaches are rotational resonance (RR) and rotational echo double resonance (REDOR) NMR. The RR NMR approach selectively restores the dipolar couplings by spinning the sample such that an integral multiple of the MAS frequency is

equal to the chemical shift difference ($\Delta\omega$) between two NMR resonances [16-18]. The distance limits for $^{13}C...^{13}C$ measurements using RR NMR is ~6.5 Å with resolution on the order of 0.3 Å. The REDOR NMR approach has been developed to measure weak heteronuclear dipolar couplings, such as those between ^{31}P and ^{13}C or ^{15}N and ^{13}C [19,20]. REDOR relies on the dephasing of magnetization of the observed spin through coupling to a second spin. The distance limit for $^{13}C...^{15}N$ measurements using REDOR NMR is ~5 Å with resolution on the order of 0.2 Å.

The global secondary structure and orientation of membrane proteins and peptides can be probed by polarized FTIR spectroscopy using the amide I vibration as a structurally sensitive marker [21]. The frequency of the amide I mode depends on hydrogen-bonding of the C=O group as well as on the geometry of the peptide backbone. Bands centered at ~1654 cm^{-1} correspond to α-helical structures, while bands centered at 1624 - 1637 cm^{-1} and 1675 cm^{-1} correspond to the out-of-phase and in-phase modes of β-sheet structures, respectively or alternatively β-turn. Fourier self deconvolution (FSD) of the amide I region [22] can yield quantitative estimates of the relative ratios between the different secondary structural elements of the protein [23]. The orientation of C=O group, which dominates the amide I vibration, can be derived from the relative absorption of IR light polarized parallel or perpendicular to the C=O transition dipole moment. Maximum absorption occurs when the polarization of light is parallel to the transition moment. In an α-helix, the C=O transition dipole is known to be oriented at an angle of ~39° relative to the helix axis [24-26].

Using both MAS NMR and FTIR, we have shown that peptides 38 residues in length that incorporate the transmembrane domain of the activated receptor (neu*TM) form extended helical structures oriented perpendicular to the membrane surface. Furthermore, the pKa of the E_{664} carboxyl group in membranes is considerably higher than that of free glutamate indicating the E_{664} carboxyl group is buried in the membrane interior, possibly in the dimer interface between TM domains.

BODY

Figure 1 (left) presents the Fourier self deconvolutions of polarized ATR-FTIR spectra of neu*TM incorporated into DMPC bilayers. The spectra were obtained with parallel (solid lines) and perpendicular (dashed lines) polarized light as described in [16]. The amide I band is centered at 1655 cm^{-1}, characteristic of helical secondary structure. Increasing the pH from pH 5.5 to pH 7.0 results in a substantial increase in a band at 1630 cm^{-1} that may be attributed to extended β-structure. The orientation of the helical region of the peptide is estimated from the dichroic ratio (Iparallel/Iperpendicular) of the amide I band at 1655 cm^{-1}. The measured dichroic ratio at pH 5.5 corresponds to a helix angle of 25° relative to the bilayer normal. The orientation is roughly the same at pH 8.5.

The neu*TM peptides were synthesized using solid-phase methods at the Keck Peptide Synthesis Facility at Yale University. The sequence of the 38-residue peptide corresponds to residues 649-686 in the human neu receptor protein.

AEQRASPVTFIIATV-E664-GVLLFLALVVVVGILIKRRRYK

The lyophilized peptide was dissolved in trifluoroacetic acid and purified using a 5 ml POROS-R1 reverse phase high performance liquid chromatography (RP-HPLC) column (Perceptive Biosystems, Cambridge, MA) equilibrated with 95% H_2O, 2% acetonitrile and 3% 2-propanol. Peptide elution was achieved with a linear gradient to a final solvent composition of 5% H_2O, 38% acetonitrile and 57% 2-propanol. All solvents contained 0.1% trifluoroacetic acid. Fractions containing peptides were then lyophilized and assessed for purity by amino acid analysis (correlation coefficients of > 0.95) and mass spectrometry.

Peptide fractions were lyophilized and reconstituted into lipids as follows. Peptide and lipids were codissolved at a 30:1 molar ratio with 2% n-octyl β-glucopyranoside (Sigma, St. Louis, MO) in trifluoroethanol. Lipids (Avanti Polar Lipids, Alabaster, AL) used were dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylserine (DMPS) (14% by weight). Samples were dried with nitrogen gas and resolubilized in 6 ml of dialysis buffer [0.5 mM sodium azide, 0.5 mM HEPES, pH 7.0]. Samples were split into three dialysis groups for each pH. One of each lipid sample was placed into each of the following buffers: 1 mM MES, 0.5 mM sodium azide, pH 5.5; 1mM HEPES, 0.5 mM sodium azide, pH 7.0; and 1 mM TAPS, 0.5 mM sodium azide, pH 8.5; and dialysed overnight. 300 ul of each sample were allocated for ATR-FTIR, approximately 100 ul used for transmission IR, and the remaining pelleted and prepared for MAS-NMR experiments.

FTIR spectra were recorded on a Nicolet Magna 550 spectrometer purged with N_2 (Madison, WI) and equipped with a MCT/A detector. For transmission spectra, typically 50 µl of sample (protein concentration of 36 - 90 mM) is dried on AgCl windows with dry air. For polarized ATR-FTIR spectra, the spectrometer is equipped with a KRS-5 wire grid polarizer (0.25 mm spacing, Grasbey Specac, Kent, UK). The sample (~300 µl, 36 - 90 mM) is dried on the surface of a Ge internal reflection element and placed in a variable angle ATR accessory (Grasbey Specac, Kent, UK). Fourier self deconvolution spectra [FSD, 22] were obtained using a bandwidth of 13 cm^{-1} and an enhancement factor of 2.4, determined by Byler and Susi [23] to best fit experimental data. The helical content and orientation was determined using the approach described in our recent work on phospholamban [9].

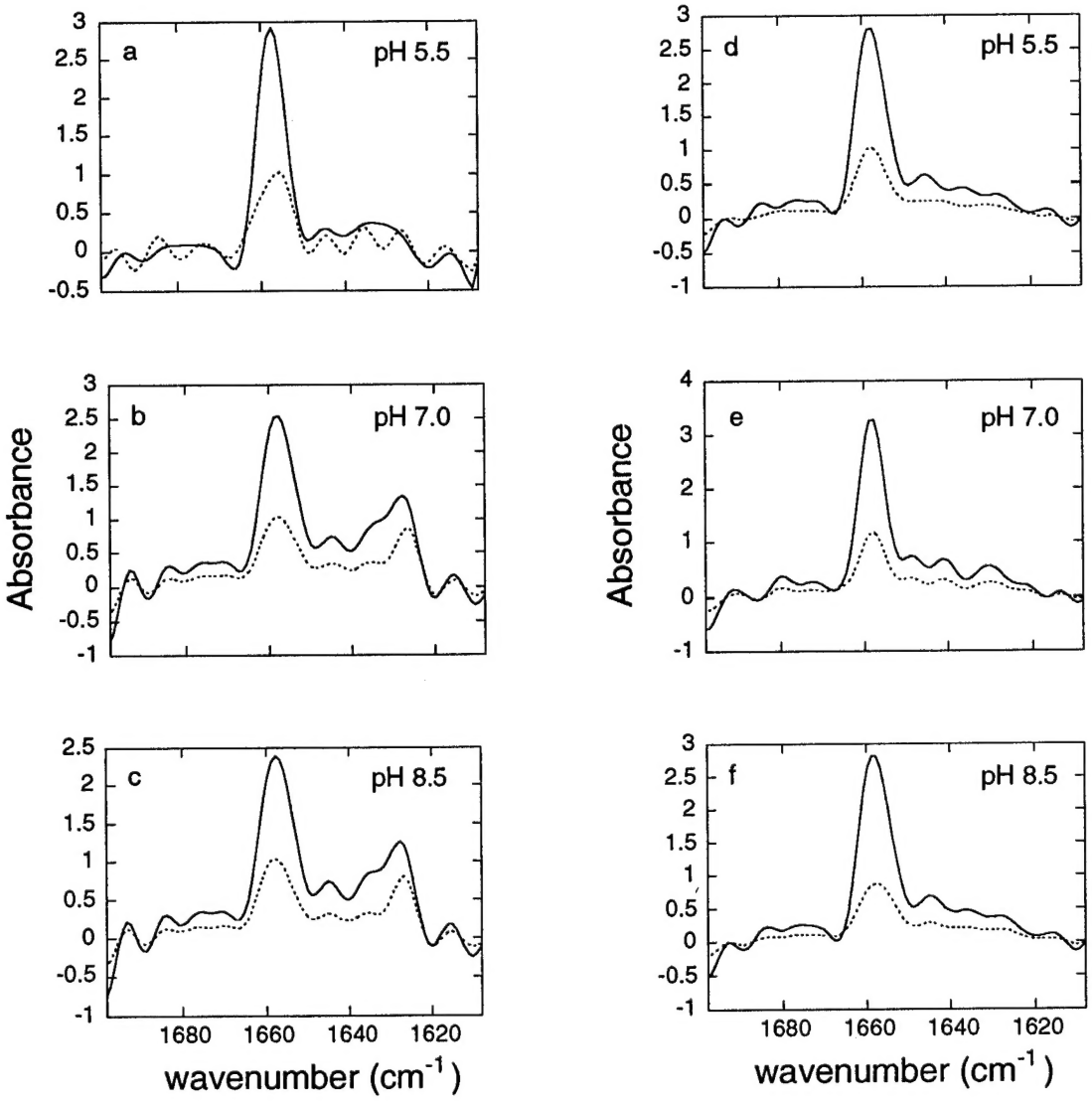

Figure 1: (Left) Polarized FTIR spectra of the amide I band of neu*TM in DMPC multilayers. Fourier self deconvolutions are shown of spectra obtained of oriented membranes at pH 5.5 (a) pH 7.0 (b) and pH 8.5 (c). Spectra were obtained with parallel (solid) and perpendicularly (dashed) polarized light.

(Right) Polarized FTIR spectra of the amide I band of neu*TM in DMPC:DMPS multilayers. Fourier self deconvolutions are shown of spectra obtained of oriented membranes at pH 5.5 (d) pH 7.0 (e) and pH 8.5 (f). Spectra were obtained with parallel (solid) and perpendicularly (dashed) polarized light.

The most straightforward interpretation of the FTIR data is that increasing the pH results in local unfolding of the peptide. The current hypothesis is that protonation of E_{664} allows the peptide to fold into a long α-helix spanning the membrane stretching from the C-terminus through position 664. Deprotonation is thought to lead to unfolding of the helix from E_{664} to the N-terminus of the protein. This suggests that E_{664} titrates with a pKa between 5.5 and 7. This is a substantial increase over the 4 - 4.5 solution pKa of glutamic acid. However, an increased pKa might be expected to result from the surface charge on DMPC bilayers. Previous studies on membrane lipids and fatty acids have shown that the apparent pKa increases near the membrane surface [28-30]. The apparent pKa corresponds to the bulk pH at which half of the COOH groups are protonated, and should be distinquished from the intrinsic pKa which corresponds to the surface pH at which half of the COOH groups are protonated.

In order to determine whether the E_{664} side chain titrates with a pKa between 5.5 and 7.0, MAS NMR spectra were obtained of neu*TM labeled with [5-^{13}C] glutamate reconstituted into DMPC bilayers. The ^{13}C chemical shift of the E_{664} carboxyl group is sensitive to its protonation state with the isotropic chemical shift moving downfield upon deprotonation [27].

Figure 2 presents MAS NMR spectra of the neu*TM domain in DMPC bilayers at pH 5.5 (a), pH 7.0 (b) and pH 8.5 (c). In Figure 2a, the resonance at 179.6 ppm is assigned to the side chain carboxyl of E_{664} based on a comparison with unlabeled peptide. Increasing the pH to 7.0 (Figure 2b) results in the appearance of a second resonance at 180.9 ppm. The intensity of the 180.9 ppm resonance increases in intensity when the pH is further increased to 8.5 (Figure 2c). Integration of the intensities of a full pH series from pH 5.5 to 8.5 indicates that the apparent pKa of E_{664} in DMPC bilayers is ~6.5. Again, this is a substantial increase over the 4 - 4.5 solution pKa of glutamic acid.

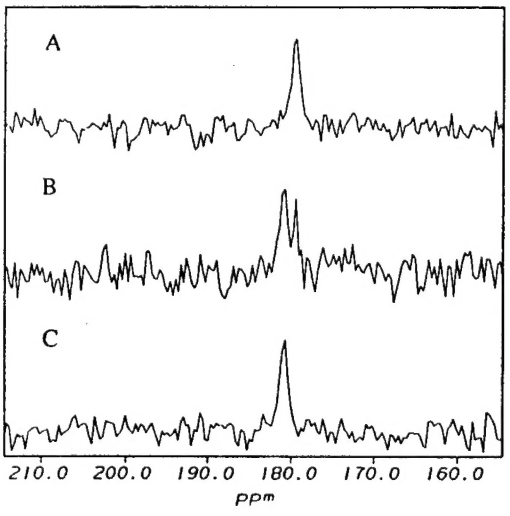

Figure 2: MAS NMR spectra of neu*TM at pH 5.5 (a), 7.0 (b) and 8.5 (c) in DMPC multilayers. Spectra were obtained at a ^{13}C frequency of 90.4 MHz with a MAS speed of 5.000 kHz. The spectra shown are actually difference spectra where a spectrum of unlabeled neu reconstituted in DMPC has been subtracted. The temperature was maintained at -20°C. At higher temperatures, the two resonances broaden and collapse into a single line due to exchange.

In order to establish how membrane surface charge influences the pKa of the E_{664}, we incorporated 14% DMPS into the DMPC vesicles. DMPS is the major negatively charged lipid in mammalian cell membranes and usually ranges in concentration from 10 - 20%. The FSDs of the amide I band of neu*TM in DMPC:DMPS membranes are shown in Figure 1 (d-f). The frequency and intensity of the FSD band at 1655 cm^{-1} argue that the peptide is predominantly helical. The orientation is significantly more transmembrane than in DMPC vesicles.

Figure 3 presents a model that incorporates the results described above. First, the region C-terminal to position 664 is helical and oriented roughly perpendicular to the membrane plane. When the E_{664} carboxyl group is deprotonated, the region N-terminal to position 664 unfolds and the COO^- group is exposed to the polar membrane interface. Protonation allows the peptide to adopt a helical conformation oriented perpendicular to the membrane plane. The pKa of the E_{664} side chain is shifted by the membrane surface charge. Under conditions approximating those in native membranes, the carboxyl group readily partitions into the membrane. The high pKa observed for the E_{664} carboxyl group and increased orientation in DMPC:DMPS membranes may reflect dimerization of the peptides in membranes.

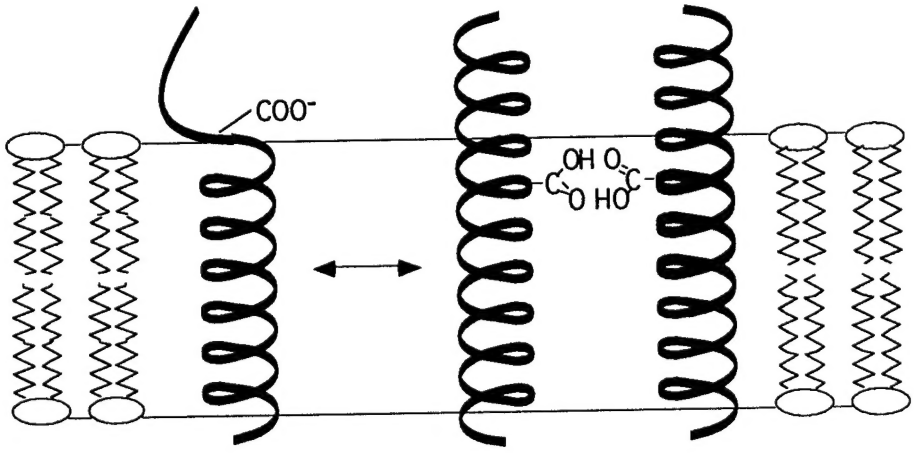

Figure 3: Model illustrating the influence of membrane surface potential on the pKa of E_{664} in neu*TM.

CONCLUSIONS

In general, hydrogen-bonding interactions between amino acid side chains or between side chains and the helix backbone promise to be a major mechanism for gaining specificity in dimer formation. The results on neu*TM described above set the stage for studying hydrogen-bonding interactions neu*TM helices. We have shown that the neu*TM peptides form extended helical structures oriented perpendicular to the membrane surface. Furthermore, the high pKa of the COOH group in neu*TM reconstituted into DMPC:DMPS membranes indicate that the E664 carboxyl group is buried in the membrane interior, possibly in the dimer interface between TM domains.

The experimental plan is to test two models for hydrogen-bonding interactions in the dimer interface of neu*TM (Figure 4). We plan to study both glutamic acid and glutamine at position 664. Both substitutions are known to activate the receptor. Studies on glutamine have an advantage in that heteronuclear experiments can be designed using ^{15}N and ^{13}C labels. The transmembrane peptides containing glutamine are also not sensitive to the membrane surface charge. This removes surface charge as a variable in characterizing dimer structure. The first model involves side chain - side chain hydrogen bonding interactions as the mechanism for receptor dimerization. The second model involves backbone - side chain interactions.

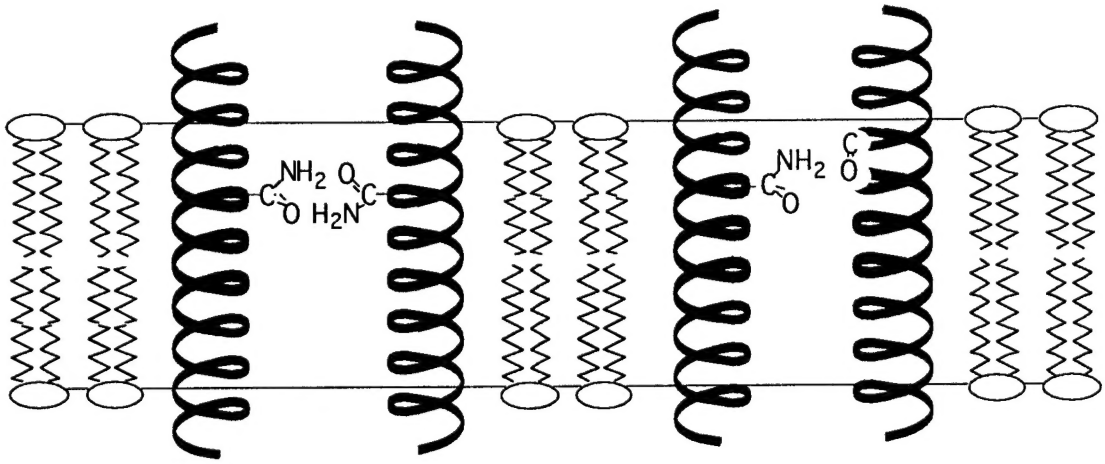

Figure 4: Models for neu*TM dimerization. In (a), dimerization is mediated by hydrogen-bonding interactions between the polar side chains of glutamine residues. In (b), dimerization is mediated by hydrogen-bonding interactions between backbone carbonyls and the polar side chain of glutamine.

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